## Title

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# Identification and Purification of an Endogenous Receptor for the Lectin Pallidin from *Polysphondylium pallidum*

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ABSTRACT We report the identification and purification of an endogenous carbohydrate-containing receptor of pallidin, the cell surface lectin implicated in mediating cell-cell adhesion in the cellular slime mold *Polysphondylium pallidum*. The receptor is identified in an aqueous extract of crude *P. pallidum* membranes as a potent inhibitor of the hemagglutination activity of pallidin. The inhibitor is purified to apparent homogeneity by affinity precipitation with pallidin followed by fractionation of the solubilized precipitate on Sepharose 4B. The hemagglutination inhibitor (HAI) is metabolically radiolabeled, indicating that it is a biosynthetic product of the amoebae and not an ingested food substance. The HAI is released into the extracellular medium by living, differentiated amoebae. This release is markedly facilitated by the addition of D-galactose, a specific saccharide that binds to pallidin. Hence, the HAI appears to have an *in situ* association with pallidin at the cell surface. Exogenously added HAI promotes the agglutination of differentiated amoebae in a gyrated suspension at very low concentrations. The results are consistent with a model of cell-cell adhesion in which the HAI is a multivalent, extracellular aggregation factor that is recognized by pallidin molecules on adjacent cells. The HAI would then be analogous to the aggregation factors identified in marine sponges.

Aggregation-competent amoebae of the cellular slime mold *Polysphondylium pallidum* contain a soluble galactose-binding hemagglutinin (1). This lectin, pallidin, consists of a family of three closely related isolectins, each consisting of different combinations of three subunits of 25–27 kdaltons (2, 3). Considerable evidence indicates that this lectin may be involved in intercellular adhesion of the slime mold amoebae: (*a*) the lectin is present in an active carbohydrate-binding form on the cell surface of differentiated, mutually adhesive amoebae and in a lesser amount on vegetative amoebae (1, 4); (*b*) high-affinity receptor sites for pallidin are present on the cell surface of differentiated amoebae, as detected by agglutination studies, binding measurements, and cytochemical means (1, 4, 5); (*c*) high concentrations (>50 mM) of specific saccharide inhibitors of pallidin (lactose or D-galactose) selectively block the cohesiveness of amoebae in swirled suspensions (1); (*d*) the specific lectin antagonists, asialofetuin and immune F'ab, also block the intercellular adhesion of gyrated amoebae when the assays are carried out in hypertonic media or in the presence of antimetabolites (6, 7). These results suggest that the complementary interaction between pallidin and its receptor on adjoining cells is involved in the mediation of cell-cell adhesion. Recent genetic experiments have established that the principal cell surface lectin of another species, *Dictyostelium discoideum*, is required for cell adhesion (8, 9). Other ligand-receptor systems may also participate in adhesion of *P. pallidum* cells, as suggested by the immunological identification of cell surface antigens that elicit adhesion-blocking antibodies (10, 11). These antigens are apparently distinct from pallidin, but no evidence exists on their relationship to the receptors for pallidin.

Here we focus on the identification of the endogenous carbohydrate-containing receptor for pallidin. We report the purification of a pallidin-binding glycoconjugate which is a major component of differentiated amoebae. We provide evidence that this water-soluble substance is associated with the cell surface via an interaction with pallidin. This cell surface association and the specific aggregation-promoting activity of the glycoconjugate in cell reaggregation assays suggest that the glycoconjugate is an extracellular aggregation factor analogous to that found in the marine sponge system (12–14).

## MATERIALS AND METHODS

### *Materials*

Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Dextran sulfate (mol wt 8,000), heparin (Grade I), guanidine hydrochloride

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(Grade I), 2,4 dinitrophenol, D-galactose, D-mannose, fetuin (type III), α-chymotrypsinogen A, bovine serum albumin (BSA), cytochrome *c* (type III), and ovalbumin (grade VI) were obtained from Sigma Chemical Co. (St. Louis, MO). Fetuin was desialylated by the procedure of Spiro (15). $^3$H-acetate (2.0 Ci/mmol sp act) came from New England Nuclear (Boston, MA). Medium components for culturing *P. pallidum* were obtained from Difco Laboratories (Detroit, MI). All reagents for SDS PAGE were obtained from Bio Rad Laboratories (Richmond, CA). The zwitterionic detergent, Empigen BB, was a gift from Albright and Wilson, Ltd. (Whitehaven, England). All other chemicals were of reagent grade.

## Cell Culture

*P. pallidum*, strain WS 320, was used in all experiments. Cells were grown either on agar surfaces in association with *Klebsiella aerogenes* or in suspension in association with autoclaved *E. coli B/r* (obtained from American Type Culture Collection, Rockville, MD). For surface-grown cells, *K. aerogenes* was pregrown to stationary phase in HL5 medium (16) containing bacto-peptone instead of proteose peptone #2. $1.2 \times 10^6$ *P. pallidum* spores in 3 ml of the pregrown culture of *K. aerogenes* were inoculated onto 1-cm deep SM nutrient agar (17) layers in disposable aluminum foil pans (25 × 30 × 4 cm). The pans were covered with aluminum foil and kept in a moist, dark atmosphere at 22°C. The amoebae deplete the bacteria and become cohesive by 4 d. The cells do not aggregate and complete morphogenesis to sorocarps unless exposed to light. For large-scale purifications, the cells ($10^{10}$/pan) were harvested after 5 d of incubation in the dark. At this stage, the cells were still amoeboid with a very low percentage of microcysts. For the cell cohesiveness assays (see below), small preparations of cells were obtained by inoculating $10^5$ spores in 0.25 ml of bacterial suspension onto SM agar Petri plates (100 × 20 mm). The cells were harvested after 4 d of incubation in the dark.

For suspension cultures, *E. coli* B/r was grown to stationary phase in modified HL5 as above. The bacteria were washed several times in SPS buffer (16.7 mM $Na_2H$–$KH_2PO_4$, pH 6.0), adjusted to an optical density of 0.490 at OD 450 when diluted 1:20, and then autoclaved for 20 min. 50-ml aliquots of bacterial suspension in 125-ml Erlenmeyer flasks were inoculated with $10^5$ spores/ml. Streptomycin sulfate and penicillin G were added at 100 μg/ml and 100 U/ml, respectively. The flasks were swirled at 200 rpm at 25°C on a New Brunswick G24 gyratory shaker (New Brunswick Scientific Co., Edison, NJ). Cells were harvested during the growth phase at densities of $7 \times 10^6$ cells/ml or less and separated from bacteria by repeated washing and centrifugation (500*g*, 5 min). The cells were then differentiated by swirling ($10^7$ cells/ml) at 200 rpm (25°C) in SPS containing penicillin and streptomycin. The amoebae were metabolically labeled during differentiation by adding $^3$H-acetate at 10 μCi/ml.

## Hemagglutination Inhibition Assay

The hemagglutination activity of pallidin was assayed as previously described with formalinized human O(F-0) erythrocytes (1). A mixture of the pallidin isolectins was purified by affinity chromatography on acid-treated Sepharose 6B with elution by D-galactose (3). Each well of the microtiter V-plate (Cooke Engineering, Alexandria, VA) contained 25 μl of a serial twofold dilution of pallidin in NaCl/Pi buffer (15 mM $NaH_2$–$Na_2H$ $PO_4$, 0.25 M NaCl, pH 7.2), 25 μl of F-0 cells in Pi buffer (75 mM $Na_2H$–$KH_2PO_4$, 75 mM NaCl, pH 7.2) and 25 μl of NaCl/Pi buffer with or without inhibitors. The titer was determined after 1 h as the reciprocal of the highest pallidin dilution giving positive agglutination. The end-point concentration of pallidin was ~1 μg protein/ml. A unit of inhibitor was defined as the quantity that reduced the titer of pallidin by 75% (two full steps) relative to the control. Inhibitors were tested at a series of twofold dilutions to determine the dilution corresponding to 1 U.

## Purification of Hemagglutination Inhibitor (HAI)

The starting cells were either surface-grown or suspension-grown as described above. An acetone powder was prepared from a crude particulate fraction of these cells. First, the cells were harvested and washed in cold distilled water with centrifugation at 1,500 rpm for 10 min (JA-14 rotor, Beckman J21B centrifuge; Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The cells were pelleted at 4,000 rpm for 10 min and then frozen in liquid nitrogen for 5 min. The thawed pellet was suspended in cold 0.15 M NaCl ($10^8$ cells/ml) and spun at 20,000 rpm for 30 min in the JA-20 rotor. This wash step was repeated and the pellet was then homogenized in 10 volumes of acetone (−10°C). The precipitate that formed was filtered out on Whatman #1 paper and allowed to dry. To solubilize the HAI, the acetone powder (AP) was homogenized and boiled in NaCl/Pi (10 mg of powder/ml) for 5 min. The soluble AP extract (20,000 rpm, 30-min supernatant) contained 128–512 U of hemagglutination inhibitory activity per milliliter. The HAI was precipitated by the addition of purified pallidin. 1 vol of AP extract was mixed with 0.5–3 vol of pallidin (200–300 μg/ml) that had been dialyzed for 4 h against NaCl/Pi containing 0.5% Empigen BB. The presence of the detergent helped to maintain the solubility of the pallidin. After 14–16 h at 4°C, the precipitate that formed was spun out at 20,000 rpm for 45 min. The precipitate was solubilized by boiling in guanidine hydrochloride (6 M) for 10 min and then was fractionated on a column of Sepharose 4B equilibrated in NaCl/Pi. Fractions were analyzed for HAI inhibitory activity, neutral sugar and protein (OD at 280 nm). Unless otherwise noted, all neutral sugar analyses were done by the phenol-sulfuric method (18) using glucose as a standard. Radiolabeled fractions were counted with Hydrofluor (National Diagnostics, Inc., Advanced Applications Institute, Inc., Somerville, NJ) in a Beckman Liquid Scintillation Counter.

## Conditioned Medium

4-d surface grown *P. pallidum* cells were harvested from pans and washed as described above and then suspended at $10^8$ cells/ml in SPS with or without sugars. The cells were swirled at 200 rpm on the G24 Shaker for varying times and then centrifuged out at 2,000 rpm for 7 min. Cells were shown to be at least 98% viable by trypan blue exclusion at the time of harvest. A final supernatant which we refer to as conditioned medium was obtained by centrifugation at 20,000 rpm for 30 min. Conditioned medium was concentrated by ultrafiltration on a PM-30 filter (Amicon Corp., Scientific Systems Div., Lexington, MA). It was then boiled for 10 min and dialyzed against NaCl/Pi. Purification of the HAI in the conditioned medium employed the same steps as described above for the AP extract.

## Electrophoresis

Discontinuous SDS electrophoresis was carried out according to Laemmli (19) in 10% slab gels. BSA (68,000), chymotrysinogen A (25,700), ovalbumin (43,000) and cytochrome *c* (12,000) were run as molecular weight standards. The gels were stained in 0.2% Coomassie Blue (G-250) in methanol/water/acetic acid (50:50:10) for 20 min. The gel was destained in 7.5% acetic acid, 5% methanol. Dilute samples were precipitated with 10% TCA for 30 min (4°C), after which the precipitates were spun out at 10,000 rpm for 15 min. Before solubilization the pellets were washed three times with absolute ethanol, centrifuging as above after each wash.

Cellulose acetate electrophoresis was performed in a Gelman electrophoresis box using Cellogel 500 (Kalex Scientific Co., Inc., Manhasset NY). Samples were concentrated after dialysis into water by drying under nitrogen and air. 2- to 4-μl vol were loaded onto strips presoaked for 30 min in the running buffer. The cellulose acetate strips (5 cm × 16.8 cm) were run at 12.5 mAmps for 20 min or 1.5 h in pH 3.5 pyridine-acetic acid buffer. To test other pH's, Pi buffer (pH 7.2), 0.05 M LiCl in 0.01 N HCl (pH 2.0), and 0.1 M barium acetate buffer (pH 8.6, 0.05 ionic strength) were also employed as running buffers. The strip was stained for 15 min in 1% Alcian blue in 2.5% acetic acid and destained in 2.5% acetic acid. In some runs, the strips were treated with periodic acid (20) before staining. In other runs, the strips were stained with Amido black by soaking the strip in a large volume of 0.1% Amido black in 7% acetic acid for 30 min and destaining in 2.5% acetic acid, 40% ethanol. Metabolically labeled material was run for 1.5 h in pyridine-acetic acid buffer (pH 3.5) and analyzed by cutting the unstained lane into 2-mm strips. Each strip was placed in 1 ml of water in a counting vial, shredded, and shaken for 1 h. Hydrofluor scintillation cocktail (10 ml) was added to each vial and the vials were counted.

## Cohesiveness Assay

4-day, surface-grown *P. pallidum* cells were harvested from agar plates and washed in cold distilled water as described above. The amoebae were suspended at $3.2 \times 10^7$ cells per ml in EDTA/Pi (16.7 mM $Na_2H$–$KH_2PO_4$, 10 mM EDTA, pH 6.2) containing 4 mg/ml of bovine serum albumin (BSA). The cell suspension was dispersed into single cells by repeated pipetting with a Manostat (Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL) and was combined (0.5 ml) with 0.5 ml of 10 mM 2,4 Dinitrophenol (DNP) in EDTA/Pi and 1 ml of EDTA/Pi with or without glycoconjugates in 10-ml Erlenmeyer flasks. The flasks were gyrated at 125 rpm on the G-24 shaker at 25°C. 50-μl aliquots of the cell suspensions were taken at various times and carefully diluted into 10 ml of EDTA/Pi. The degree of cell agglutination was monitored with a Coulter Electronic Particle Counter (Coulter Electronics, Inc., Hileah, FL). The number of cell aggregates was determined by setting the upper threshold at infinity and the lower threshold to exclude single cells. The change in aggregates (Δ aggregates) was calculated as the number of aggregates at time (*t*) minus the number of aggregates at time-zero. We found that maximal aggregation occurred by 30 min.

## RESULTS

### *Purification*

As a source of receptor, we started with an acetone powder prepared from a crude membrane fraction of aggregation-competent amoebae. The acetone powder was extracted in NaCl/Pi buffer with boiling to release glycoconjugate receptors that were complexed with pallidin. The soluble extract, obtained after centrifugation, contained a potent inhibitor of the hemagglutination activity (HA) of pallidin. As shown in Table I, 0.8 μg/ml (neutral sugar) of this crude extract inhibited the HA of pallidin by 75%, whereas 5,700 μg/ml (neutral sugar) of lactose was required to inhibit the HA to the same extent. This crude extract also blocked the interaction of $^{125}$I-asialofetuin with pallidin (data not shown). Extraction of the AP in NaCl/Pi with detergent (0.5% Empigen BB) resulted in identical HA inhibitory activity, indicating that, within the limits of the assay, detergent did not release any additional inhibitor from the particulate fraction.

Initially, we attempted to purify the inhibitor by conventional affinity chromatography on a column of pallidin covalenty coupled to Sepharose. This approach was unsatisfactory, because recovery of activity was only ~10% (data not shown). A more useful technique was lectin affinity precipitation. We found that addition of purified pallidin to the AP extract resulted in the formation of a fine precipitate. With the addition of increasing amounts of pallidin to a fixed amount of extract, the amount of neutral sugar in the precipitate increased concomitant with the disappearance of hemagglutination inhibitory activity from the supernatant (Fig. 1). The precipitation depended on the carbohydrate-binding activity of pallidin since D-galactose (0.3 M) blocked precipitate formation while D-glucose (0.3 M) did not. After a precipitation that removed 75% of the inhibitory activity, the SDS gel protein patterns of the supernatant (SN) and the starting extract (AP) were indistinguishable except for the augmented pallidin bands in the former (Fig. 2).

The pallidin precipitate was analyzed by solubilizing in boiling guanidine hydrochloride and fractionating on a column of Sepharose 4B. Two major peaks of protein (OD 280), labeled pools *1* and *3* in Fig. 3, and two major peaks of carbohydrate (neutral sugar), labeled pools *2* and *4*, were resolved. The same basic profile was observed when the precipitate was solubilized in boiling SDS (not shown). SDS gel analysis established that the two protein peaks (pools *1* and *3*) consisted of pallidin, probably denatured to different degrees by the guanidine treatment (Fig. 2, lanes *1* and *3*). Almost all of the precipitated HA inhibitory activity was recovered in the first major carbohydrate peak (pool *2*), which contained 55% of the applied carbohydrate (Table II). Pools *1*, *3* and *4* had little or no activity. The minor carbohydrate peak which comigrated with pallidin at the void volume (pool *1*) was probably due to pool *2* material complexed with pallidin. The possible sources of pool *4* are considered below.

Although pool *2* contained no Coomassie-Blue-staining bands (Fig. 2, lane *2*), periodic acid-Schiff staining revealed a diffuse band in the 3% stacking gel of SDS PAGE (data not shown). Cellulose acetate electrophoresis with Alcian blue staining revealed a single diffuse component in pool *2* that migrated slightly towards the anode from the loading line (Fig. 4). Only a single band was seen under a variety of run conditions (between pH 2.0 and 8.6; short or long electrophoresis

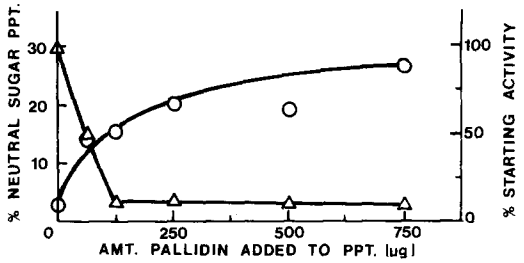


FIGURE 1 Precipitation of hemagglutination inhibitory activity by addition of pallidin. 1-ml aliquots of an acetone powder extract containing 128 U/ml of inhibitory activity were combined with various volumes of pallidin (250 μg/ml) as described in Materials and Methods. The total volume of each mixture was adjusted to 4 ml with NaCl/Pi containing 0.5% Empigen BB. After 15 h at 4°C, the precipitates were spun out at 14,000 for 45 min, washed once with 0.375% Empigen BB in NaCl/Pi, and analyzed for neutral sugar (○) by the Anthrone method (23). The supernatants were boiled for 5 min to inactivate residual lectin and then assayed for remaining inhibitory activity after dialysis into NaCl/Pi buffer. The percent of the starting inhibitory activity (△) for each supernatant is shown.

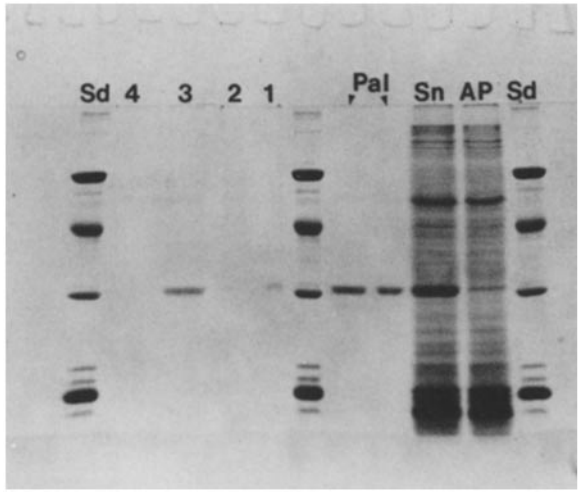


FIGURE 2 SDS PAGE analysis of purification of HAI. Fractions obtained from purification described in Fig. 3 and Table II were electrophoresed and stained for protein with Coomassie Blue as described in Materials and Methods. The lanes were loaded with the following samples: acetone powder extract (*AP*), 150 μl; supernatant after precipitation (*Sn*), 300 μl; pool *1*, ~150 μl; pool *2*, 300 μl; pool *3*, 300 μl; pool *4*, 300 μl; pallidin (PAL), 4 and 6 μg; and protein standards (*Sd*), 5 μg each of BSA, ovalbumin, chymotrypsin, and cytochrome *c*. *AP*, *Sn*, and pools *1*-*4* were concentrated by TCA precipitation before loading. Note that the only detectable proteins bands in pools *1* and *3* are pallidin subunits.

TABLE I

*Inhibition of HA of Pallidin*

| Inhibitor | Concentration to reduce HA by 75% |
|---|---|
| | *μg neutral sugar eq/ml* |
| Lactose | 5,700 |
| Asialofetuin | 0.05 |
| Acetone powder extract | 0.79 |
| Purified HAI | 0.1 |

Inhibitors were titered out as described in Materials and Methods to determine concentrations corresponding to 1 U of inhibitory activity. Concentrations are given in terms of neutral sugar equivalents. Purified HAI is pool *2* material from a Sepharose 4B fractionation (Fig. 3). The acetone powder extract is the soluble supernatant obtained after boiling the acetone powder of crude membranes in NaCl/Pi and centrifuging.

TABLE II

*Purification of the HAI*

| | Protein | Neutral Sugar | Total inhibitory units | Starting inhibitory units |
|---|---|---|---|---|
| | *mg* | *mg* | | % |
| Acetone powder extract | 9.2 | 12 | 5,100 | 100 |
| Supernatant after precipitation | 11* | 150‡ | 1,300 | 25 |
| Precipitate | 1.8 | 2 | — | — |
| Pool 1 | 0.097 | 0.076 | 190 | 4 |
| Pool 2 | <0.3 | 1.1 | 3,500 | 69 |
| Pool 3 | 0.27 | <0.4 | 54 | 1 |
| Pool 4 | 0.13 | 0.48 | | 0.2 |

The details of this purification are given in legend of Fig. 3 and Materials and Methods. The numbers given in this table for the extract, supernatant and precipitate correspond to the portion of material in the precipitate applied to the column (two-thirds of the starting precipitate). The remaining one-third of the precipitate was used for analysis. Protein concentration was determined by the Lowry method (24) with BSA as the standard.

* The extra protein in the supernatant relative to the starting AP extract is due to the added pallidin that did not precipitate.

‡ The high sugar concentration in the supernatant is due to residual free galactose that was present in the pallidin fraction.

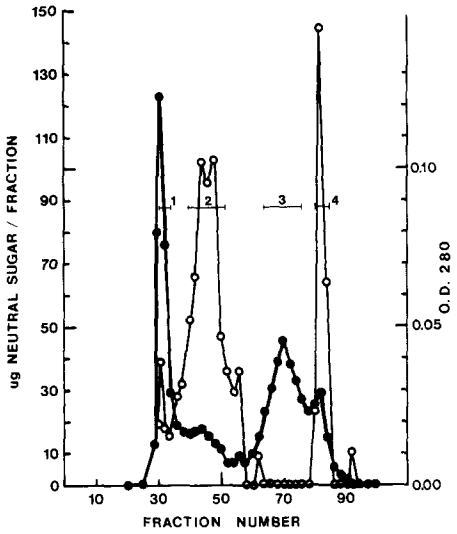


FIGURE 3 Gel filtration profile of solubilized precipitate. 30 ml of acetone powder extract derived from $2.2 \times 10^{10}$ amoebae was combined with 30 ml of pallidin (270 μg/ml) as described in Materials and Methods. The precipitate was solubilized in 3 ml of 6 M guanidine hydrochloride with boiling for 10 min. 2 ml of the solubilized precipitate was applied to a Sepharose 4B column (2.0 cm × 60 cm) at a flow rate of 20 ml/h. 2.1-ml fractions were collected and analyzed for neutral sugar (○) and OD at 280 nm (●). Four pools were made as shown in the figure. Pool *2* contained most of the applied inhibitory activity.

times; with or without periodate treatment of the paper before staining). No bands were detected by staining with amido black, a general protein stain. The component in pool *2*, which is homogeneous by cellulose acetate electrophoresis is hereafter referred to as the HAI.

The HAI was found to be a major constituent of differentiated *P. pallidum* amoebae. Approximately 2–3% of the total neutral sugar of the cells was in the form of this substance. The purified HAI was potent: 0.1 μg/ml (neutral sugar) of the HAI contained 1 unit of inhibitory activity. Lactose (on the basis of neutral sugar mass) was $6 \times 10^4$-fold less active (Table I) than

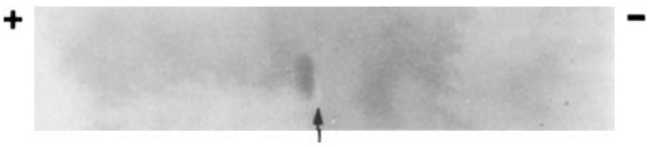


FIGURE 4 Cellulose acetate electrophoresis of HAI. Approximately 15 μg (neutral sugar) of a pool *2* such as shown in Fig. 3 was electrophoresed at pH 3.5 for 1.5 h and stained with Alcian blue as described in Materials and Methods. Periodate oxidation was not employed for the run shown but the staining pattern is identical with prior periodate-oxidation of the strip. The loading line is indicated by the arrow. The anode (+) is to the left and the cathode (−) to the right.

the purified HAI. Asialofetuin, a glycoprotein recognized by pallidin, contained approximately equivalent activity to purified HAI on a neutral sugar basis; however, on a molar basis, asialofetuin was 50- to 100-fold less active (based on a gel filtration estimate of approximately $10^6$ daltons for the molecular weight of the HAI).

A small peak of protein was coincident with the carbohydrate peak in pool *2*, indicating a possible peptide component of the HAI. Preliminary chemical characterization of the HAI revealed that it was a high molecular weight, sulfated glycoprotein rich in glucose and containing 10% peptide by amino acid analysis (D. K. Drake, S. D. Rosen and W. A. Frazier, unpublished observations).

## *Metabolic Labeling*

The *P. pallidum* amoebae used in our experiments were grown on bacteria in medium that contained complex peptone and yeast extracts. Metabolic labeling was employed to determine whether HAI was a biosynthetic product of the slime mold rather than an undegraded food substance. The amoebae were grown on autoclaved bacteria and then allowed to differentiate without food in an SPS buffer in the presence of $^3$H-acetate. Pallidin precipitation of the labeled AP extract from these cells contained a peak of radioactivity comigrating with the HAI on Sepharose 4B (Fig. 5). Furthermore, on cellulose acetate electrophoresis, a single peak of radioactivity coincided with the Alcian blue band described above (Fig. 6). Hence, during differentiation of the amoebae, the HAI was metabolically labeled. It should be noted that there was relatively little radioactivity associated with pool *4* in this experiment (Fig. 5), suggesting that pool *4* either is derived from an ingested food substance or is poorly labeled under these conditions.

## *Release of HAI into Medium*

As described above, the HAI could be extracted from a particulate fraction of differentiated amoebae. The HAI was also detected in the conditioned medium (CM) of living amoebae. When differentiated amoebae were swirled in SPS buffer at $10^8$ cells/ml, 19 U/ml of hemagglutination inhibitory activity were released after 5 h. The cells retained full viability during this period. The inhibitory activity was precipitated by the addition of pallidin. Analysis of the precipitate by 4B chromatography and cellulose acetate electrophoresis showed the presence of a component that behaved the same as the HAI. This component, like the particulate-associated HAI, could be metabolically labeled with $^3$H-acetate (data not shown). The specific activity (units of inhibitor per unit neutral sugar) of the conditioned medium was ~15% of that of purified HAI.

If the HAI were associated with the cell surface via an

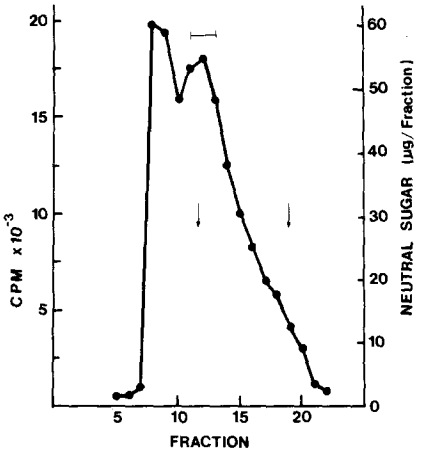


FIGURE 5 Metabolic labeling of HAI. Amoebae were grown in suspension on autoclaved bacteria and differentiated in the presence of $^3$H-acetate as described in Materials and Methods. 1 ml of a labeled AP extract (from 4 × $10^8$ cells) was precipitated with 2 ml of pallidin (275 µg/ml). The solubilized precipitate (0.5 ml) was fractionated on a 4B column (0.7 cm × 30 cm), and 50 µl of the fractions (0.55 ml) were counted for radioactivity. The total cpm per fraction is given on the y-axis (●). The peaks of carbohydrate-containing pools *2* and *4* from an unlabeled precipitate run on the same column are indicated by the arrows. The first peak of carbohydrate, corresponding to the inhibitory activity as shown above, was labeled. The ratio of cpm in pool *2* to total cpm in the starting AP extract (0.065) equals the ratio of the mass of protein and carbohydrate in an unlabeled pool *2* to that in its corresponding AP extract (0.066). Since the $^3$H from the acetate is incorporated into carbohydrates and proteins, the equivalence of the two ratios suggests that the HAI, rather than being a modified food product, is synthesized *de novo* from the same pool of precursors that go into other slime mold proteins or carbohydrates.

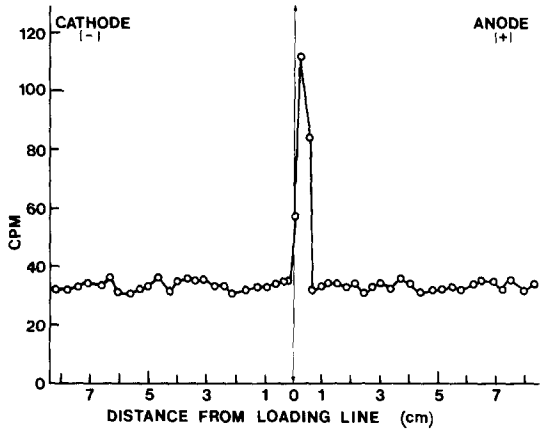


FIGURE 6 Cellulose acetate electrophoresis of labeled HAI. A pool (designated by the bar in Fig. 5) was made corresponding to the first carbohydrate peak in the 4B fractionation. 5 µl of this labeled pool was electrophoresed on cellulose acetate and counted as described in Materials and Methods. The single peak of counts which ran slightly anodally from the loading line corresponded to the Alcian-blue staining band.

interaction with pallidin, then specific saccharides recognized by the lectin should facilitate release of the HAI into the medium. Fig. 7 shows a comparison of inhibitory activity released into the medium in the presence of D-galactose (gal-CM) or D-mannose (man-CM). (The CM's were tested for activity after boiling and dialysis.) The man-CM released no more activity than SPS buffer; however, by 5 h, the gal-CM

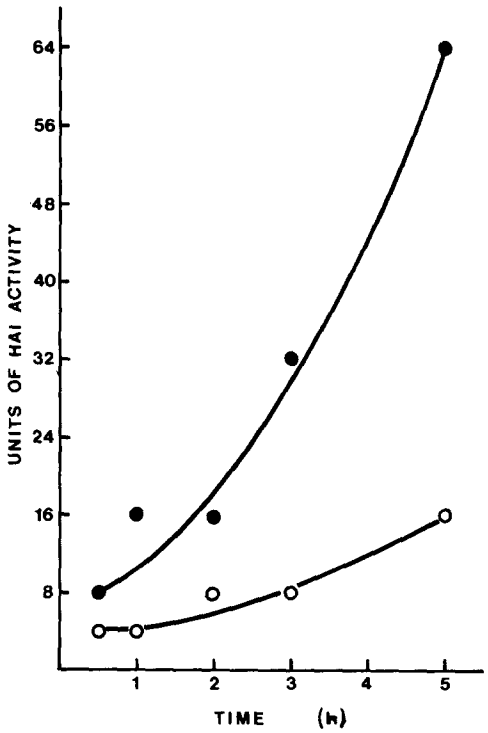


FIGURE 7 Release of HAI into conditioned medium in the presence of sugars. 65-ml aliquots of differentiated amoebae in 125-ml flasks were swirled in SPS containing 300 mM D-galactose (●) or D-mannose (○) as described in Materials and Methods. At various times, conditioned medium was collected. The cells remained 98% viable throughout the swirling. The medium was boiled, dialyzed into NaCl/Pi, and tested for hemagglutination inhibitory activity.

contained fourfold more activity than the man-CM. The inhibitor released by galactose was precipitated by pallidin and ran the same as HAI on 4B chromatography and cellulose acetate electrophoresis.

The HAI released into gal-CM represented a substantial proportion of the cells' extractable HAI. After 5 h of exposure to galactose, 64 U/$10^8$ cells had accumulated in the medium, whereas 27 U/$10^8$ cells remained in an AP extract of the cells. In contrast, cells exposed to mannose released only 16 U/$10^8$ cells into the medium and retained 94 U/$10^8$ cells that could be extracted in an AP extract of the cells. The high yield of activity in the galactose-conditioned medium may make it suitable starting material for the purification of the HAI. Attempts are underway to devise a simplified purification procedure starting with gal-CM.

## *Promotion of Cell-Cell Adhesion*

To determine its effect on cell-cell adhesion, the purified HAI was added to cells in a cell cohesiveness assay. In this assay, the number of aggregates that formed in a swirled suspension was measured quantitatively with a Coulter Counter. Under physiological buffer conditions, the HAI produced a small enhancement of cell agglutination relative to untreated controls after 30 min of gyration (data not shown). In the presence of 2,4 DNP, which reduces the endogenous cohesiveness of the amoebae (7), the HAI had significant promoting activity at concentrations as low as 0.1 µg/ml (neutral sugar), whereas control glycoconjugates (heparin, glycogen, or dextran sulfate) were inactive at concentrations up to 25 µg/ml (Fig. 8). Pool *4* was inactive at concentrations up to 10 µg/

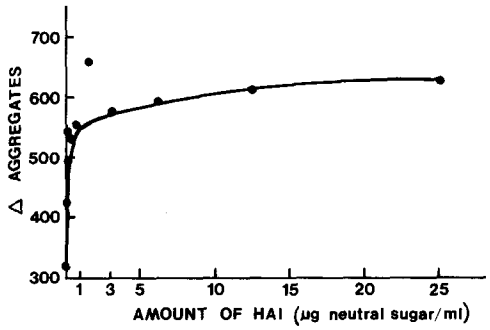


FIGURE 8 Effect of purified HAI on intercellular adhesion of *P. pallidum* amoebae. Aggregation-competent amoebae were gyrated in suspension in the presence of 2,4 dinitrophenol and various concentrations of HAI as described in Materials and Methods. After 30 min of gyration, the number of aggregates in each sample was determined with a Coulter Counter. The change in aggregates is given on the y-axis. The concentration of HAI is given in terms of neutral sugar content. Microscope examination of the samples verified that the number of aggregates as well as the size of the aggregates increased in the presence of HAI. In separate experiments, the control glycoconjugates (heparin, glycogen, and dextran sulfate) were tested. Whereas there was 160% promotion produced by 5 μg/ml HAI, glycogen produced no effect at 25 μg/ml, and dextran sulfate produced only 20% promotion at 25 μg/ml. In another experiment in which HAI produced 80% promotion at 5 μg/ml, heparin had no effect at 25 μg/ml. The control substances were chosen because their compositions are similar to that of the HAI which is sulfated and rich in glucose.

ml (neutral sugar). Microscope examination verified that the agglutinates were larger and more numerous in the presence of HAI than in the untreated controls.

## DISCUSSION

Differentiated *P. pallidum* amoebae contain a high molecular weight, water-soluble glycoconjugate that binds to pallidin and inhibits its HA. This HAI is a biosynthetic product of the cells, is found associated with a particulate fraction of the amoebae, and is also released into the medium by living cells. Extraction of the HAI from the particulate fraction does not require detergent, indicating that its association with membranes is peripheral rather than integral. The HAI is a very abundant component in differentiated amoebae, constituting ~9% of the neutral sugar in a crude particulate fraction and about 2 percent of the cells total neutral sugar. The HAI can be purified using affinity precipitation with pallidin followed by 4B gel chromatography. The HAI is homogeneous by the criterion of cellulose acetate electrophoresis. The precipitation is extremely selective, in that the only components in the solubilized precipitate besides the HAI are pallidin and an inactive carbohydrate fraction. The ability to precipitate with pallidin indicates that the HAI is multivalent.

Our observations strongly suggest that the HAI is held to the surface as a peripheral membrane component via an interaction with pallidin. Galactose, recognized by the sugar binding site of pallidin, markedly facilitates the release of the HAI from living amoebae into the medium, whereas mannose, a nonspecific sugar, releases fourfold less HAI. The HAI released into the medium by galactose represents a major portion of the particulate-associated material. Our evidence for the facilitated release of HAI from the cells by galactose and the biosynthesis of the HAI by the amoebae indicates that the HAI is neither a fortuitous inhibitor of pallidin without functional interactions nor an absorbed food substance but rather is an extracellular product that has an *in situ* association with pallidin.

Exogenously added HAI has the ability to promote the agglutination of differentiated amoebae. This effect is most clearly seen when the endogenous cohesiveness of the cells is reduced by 2,4 DNP. Presumably, the HAI being a multivalent ligand crosslinks the cells by binding to pallidin molecules on adjacent cells.

Our results are compatible with a model of cell-cell adhesion in which the HAI serves as a multivalent, extracellular receptor with carbohydrate chains that are recognized by pallidin molecules on adjacent cells. There may also be other cell surface receptors for pallidin in addition to the HAI. If pallidin is held to the cell surface by its sugar binding sites, as apparently is the case for the lectin purpurin in *Dictyostelium purpureum* (21), then there would be a class of cell surface receptors responsible for this association. Integral membrane glycoconjugates that bind to the lectin, such as those identified in *D. discoideum* by Breuer and Sui (22), would be candidates for this second class of receptors. In our model the HAI would then be analogous to the large molecular weight, proteoglycan aggregation factors that are involved in species-specific cell-cell adhesion of marine sponges (13, 14). Testing the proposed receptor function for the HAI will require preparing an antibody against this glycoconjugate and determining the effect of F'ab fragments on cell-cell interactions of the amoebae.

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